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Remarks

Claims 1-29 and 34-36 have been canceled without prejudice. Claims 30-33 and 37-42 are pending. Applicants respectfully acknowledge the allowability of claims 31-33 and 37 if written in independent format. Applicants amend claims 30 and 39 as described below. Claim 40-42 canceled herein without prejudice.

Applicants wish to thank the Examiner for discussing the rejections and applicant's evidence in an interview on October 7, 2003. Applicants have attempted to provide with this Amendment information and amendments referred to in the interview.

35 U.S.C. § 103

Claims 30, 38-42 stand rejected under section 103 as allegedly unpatentable over Chaudhary et al. in view of Neville et al., Hirsch et al., and Whitlow et al. The Office Action concedes that the scFc UCHT1-DT390 construct comprises unexpected and unpredictable properties but states that "Inventor Neville makes some attempt in his 1.132 declaration [filed with the response dated February 14, 2003] to ascribe the unexpected properties of the scFv UCHT1-DT390 construct to the UCHT1 portion, however, the Inventor provides no factual demonstration of this assertion." Thus the Examiner seems to suggest that all other constructs with truncated diphtheria toxin moieties are obvious, except for the DT390 construct.

In fact, the Declaration filed with the response dated February 14, 2003, shows data for a variety of constructs with various antibody portions and the sFv(UCHT1) constructs are preferred. The Declaration filed with the response dated February 14, 2003 also shows data for two truncated diphtheria constructs, DT389-sFv(UCHT1) (see Exhibit D attached thereto) and

DT390-sFv(UCHT1) (See Exhibit B attached thereto). These data taken together indicate that although the DT389 construct has a slightly lower toxicity than the DT390 construct, the sFV(UCHT1) moiety provides the unexpected and unpredictable properties to both DT truncation constructs. Furthermore, filed herewith is a second Declaration by Inventor Neville, which shows a variety of sFv(UCHT1) constructs with truncated diphtheria toxin moieties. Taken together these data indicate that constructs with toxin truncations, including DT389 through DT430 have high toxicity. The longer the DT moiety, the more likely circulating antibodies will reduce the effectiveness of the immunotoxin in a vaccinated individual. See specification, page 48, line 32, through page 49, line 6. The specification also teaches the effect of the circulating antibodies is due to the C-terminal 150 amino acid residue of the native toxin, which is a total of 535 residues long. Id. Thus, the optimum truncation will be from about DT385 to about DT430. Thus, although Applicants believe that claim 30 is not obvious based on the cited references, claim 30 has been amended to specify a truncation well within the range for which Dr. Neville has provided data (i.e., "about 150-145 carboxy terminal amino acid residues are truncated from the native diphtheria toxin moiety"). Support can be found at least on page 49, lines 3-12. Thus no new matter is believed to be added by this amendment.

The Office Action further states that claims 21-42 are rejected under section 103 as being unpatentable over Chaudhary et al. in view of Neville et al., Hirsch et al., Whitlow et al., and Youle. Applicants request clarification of this rejection, however, as claims 21-29 and 34-36 have been canceled without prejudice and as the Office Action states that Claims 31-33 and 37 are allowable if amended to independent format. For the reasons stated above, a variety of sFv(UCHT1) constructs with truncated diphtheria toxin moieties provided unexpected results as

compared to other immunotoxins with anti-CD3 antibody moieties. There is nothing in Youle to teach or suggest the construct of claim 30.

35 U.S.C. § 112, first paragraph

Claims 38-42 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to enable one of skill in the art to make or use the invention. Claims 40-42 are canceled herein without prejudice, and the rejection of those claims is moot.

The Examiner questions the use of immunotoxins in all diseases or conditions in which T cells are thought to play a role, questions the value of eliminating the T-cell populations, and questions to use of T-cell directed immunotoxins in certain autoimmune diseases. The specification teaches a short course of immunotoxin administration. See e.g., Example 5. This short course administration results in a transient T-cell depletion. The effect of a transient depletion is to reset the T cell population so that the T cells that are made after treatment with the immunotoxin, although genetically similar, have not experienced the environmental insult that the T cells prior to treatment have experienced. Thus, the new T cells after immunotoxin therapy are naïve. Such a transient T cell depletion is widely useful in conditions in with T cells play a role, because this enables resetting the T cell population to a pre-insult state. This is true whether the insult is an environmental cue that elicits as autoimmune disease, a transplant, or other T cell-mediated conditions or diseases.

Regarding claim 38 specifically, it should be noted that the specification provides *in vivo* data for inhibiting rejection of transplanted tissue or organs in a subject using the immunoconjugate. (See Example 7). The specification also provides *in vitro* data proving comparable toxic effects with immunotoxin fusion proteins comprising the sFv(UCHT1) and

truncated DT moieties. (See Example 9). Such data should be sufficient to prove that the immunotoxin of claim 30 is useful in inhibiting a rejection response. Applicants thus request reconsideration of the rejection.

Regarding claim 39 specifically, the inventors have published a manuscript, attached herewith as Appendix B, showing transient T-cell in a monkey autoimmune disease model using the FN18-CRM9 immunoconjugate. See Hu et al. (1997) Cellular Immunology 177:26-34. FN18 is the monkey equivalent of UCHT1 and CRM9 is a full length diphtheria toxin moiety. As monkeys are not immunized to diphtheria, the need for a truncation mutation as in DT383-430, is minimized. Furthermore, to facilitate prosecution, claim 39 has been amended to recite "a method for treating a subject with a T-cell mediated autoimmune disease." Support for this amendment can be found at least on page 53, lines 19-23 of the specification, and no new matter is believed to be added. Although the T cells direct the B cells in their immune responses, this amendment is made to address the Examiner's concern that certain autoimmune diseases are B cell mediated. Applicants, therefore, respectfully request reconsideration and withdrawn of the rejection.

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending application are believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of this application to issue.

A Credit Card Payment Form PTO-2038 authorizing payment in the amount of $950.00 for a three (3) month extension of time fee and a Request for Extension of Time are enclosed. This amount is believed to be correct; however, the Commissioner is hereby authorized to charge

any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-0629.

Respectfully submitted,

NEEDLE & ROSENBERG, P.C.

Tina Williams McKeon, J.D., Ph.D.
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CERTIFICATE OF FACSIMILE TRANSMISSION

I hereby certify that this correspondence and anything indicated as included is being transmitted via Facsimile No. 703-872-9306 addressed to: Attn: Examiner Ewoldt, U.S. Patent and Trademark Office, on the date shown below.

Tina Williams McKeon

October 22, 2003
Date

APPENDIX A

ATTORNEY DOCKET NO. 14028.0290
SERIAL NO. 09/389,565

APPENDIX A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Neville *et al.*

Serial No. 09/389,565

Filed: September 3, 1999

For: "AN IMMUNOTOXIN WITH IN VIVO T CELL SUPPRESSANT ACTIVITY AND METHODS OF USE"

Examiner: G. R. Ewoldt, Ph.D.

Art Unit: 1644

DECLARATION OF DAVID M. NEVILLE, JR. UNDER 37 C.F.R. § 1.132

Commissioner for Patents
Washington DC 20231

NEEDLE & ROSENBERG, P.C.
The Candler Building
127 Peachtree Street, N.E.
Atlanta, Georgia 30303-1811

Examiner Ewoldt:

I, David M. Neville, Jr., a citizen of the United States, residing at 9624 Parkwood Drive, Bethesda, Maryland 20814, declare that:

1. An immunotoxin fusion protein comprising the sFv portion of the monoclonal antibody UCHT1 and a truncated diphtheria toxin (DT) has surprising and unexpected properties; such as, a high relative potency when compared to other anti-CD3 monoclonal antibodies or fragments thereof in the context of the same truncated DT immunotoxins (ITs). These surprising and unexpected properties arise

from characteristics of the sFv moiety of UCHT1 that are not present in most other sFv constructs of other anti-CD3 antibodies and also arise from the unique synergy between the truncated DT and the CD3ε epitope.

2. DT390 is one diphtheria toxin truncation that retains toxic effects but also has reduced binding to circulating diphtheria toxin antibodies. DT389 is another example of such a truncation. Both sFvDT389 and sFvDT390 have toxic effects but a reduced binding to circulating diphtheria toxin antibodies.

3. Potency assays were conducted using single chain immunotoxin fusion proteins comprising various truncated toxin moieties and the sFv(UCHT1) antibody moiety. The potency assays were protein synthesis inhibition assays. The single chain immunotoxin fusion proteins were compared to a reference chemical conjugate of intact UCHT1-CRM9 (CRM9 being a DT binding site mutant) in CD3+ Jurkat cells. The data show that the toxicity effects for truncations between DT390 and DT430 are comparable. (See Exhibit 1.) Certain truncations above 430 had deleterious effects. Furthermore, as more amino acid moieties are added to carboxy terminus of the DT390, the desired effect of reducing binding to circulating diphtheria antibodies would be reduced.

4. The single chain immunotoxin fusion proteins (including sFvDT390, sFvDT410, and sFvDT430) used in the experiment shown in Exhibit 1 were expressed in a reticulocyte lysate system, and folding was therefore not optimized. The data in Exhibit 1 indicate that the single chain immunotoxin fusion proteins are less toxic than the chemically conjugated immunotoxin (labeled IT), but these data reflect the improper folding of the expressed fusion protein in the reticulocyte lysate system. The data shown with my previous declaration indicate that potentcy for properly folded DT390sFv(UCHT1) constructs are comparable to the immunoconjugate.

ATTORNEY DOCKET NO. 14028.0290
SERIAL NO. 09/389,565

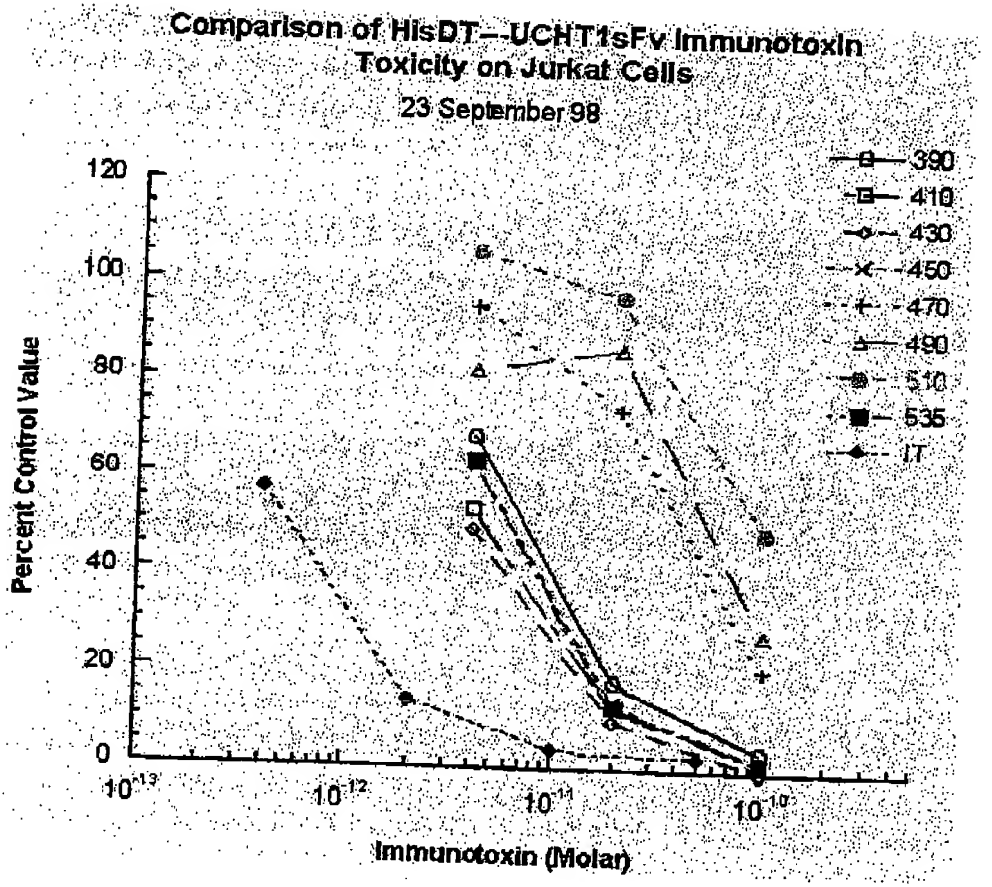
5. The data shown in my previous declaration indicates that immunotoxin fusion proteins with truncated diphtheria moieties that comprise less than 390 residues retain the desired toxic effect. Specifically DT389, has slightly less toxicity but retains the reduced diphtheria toxin antibody binding as compared to the native toxin moiety. Thus, immunotoxins that include truncated diphtheria moieties comprising about 390 residues (or, more specifically, 385-430 residues) would demonstrate the desired characterisitic – toxicity and reduced antibody binding.

6. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or document or any patent issuing therefrom.

10-20-03
Date

David M. Neville, Jr., M.D.

APPENDIX A
EXHIBIT 1



APPENDIX B

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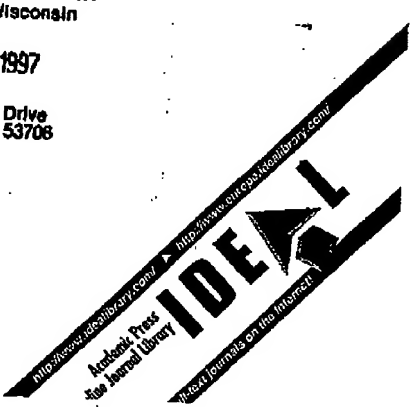
HSL

Cellular Immunology

Editor-in-Chief
Ethan M. Shevach



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ARTICLE NO. CI971096

Depletion of T Lymphocytes with Immunotoxin Retards the Progress of Experimental Allergic Encephalomyelitis in Rhesus Monkeys

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FN18-CRM9 is an anti-rhesus anti-CD3 immunotoxin that can transiently deplete T cells to 1% of initial values in both the blood and lymph node compartments and can induce long-term tolerance to mismatched renal allografts. We have investigated the ability of this immunotoxin to interdict the course of an experimental rhesus T-cell-driven autoimmune disease, experimental allergic encephalomyelitis (EAE) induced by myelin basic protein. Monkeys showing CSF pleocytosis were then treated with FN18–CRM9 alone or in combination with cranial irradiation (325 or 650 cGy). EAE in nontreated control monkeys progressed rapidly. Paralysis occurred 4–6 days after CSF pleocytosis. Paralysis was either delayed or never occurred in treated monkeys, and histopathology revealed few inflammatory plaques that were notable for their low or absent T cell content. While T cells repopulate in the periphery posttreatment, they do not return to the CNS in large numbers, suggesting that the newly repopulated T cells have lost their previously acquired CNS homing capability. Anti-CD3 immunotoxin may be useful in treating clinical T-cell-driven autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is an inflammatory T-cell-mediated autoimmune disease of the central nervous system that can be induced in a number of species by immunization with CNS components such as myelin basic protein (MBP) and adjuvant (1–6). In humans, MBP-reactive T cells are thought to initiate acute disseminated encephalomyelitis that follows infection or vaccination (4). EAE has also served for the testing of therapies for multiple sclerosis with variable predictive results (7). EAE is a far from perfect model for multiple sclerosis. In part this is due to the variability of the model in different genetic backgrounds, observed in both inbred rodents and outbred nonhuman primates. In the latter case the disease ranges between monophasic and self-limiting, relapsing-remitting and monophasic hyperacute. However, more than any other disease model, EAE has contributed to current concepts of T-cell-mediated organ-specific immunity and has permitted a dissection of immune effector and suppresser mechanisms (7).

T lymphocytes play a central role in the induction phase of EAE. This has been demonstrated by adoptive transferring of MBP-reactive T cells to induce EAE in naive syngeneic rodents and in unimmunized *Callithrix jacchus* marmoset (1, 2, 4). Moreover, it has been shown recently that T cell receptor transgenic mice specific for MBP develop spontaneous EAE at a very high incidence (8). However, it is less clear what part T cells play in the ongoing disease, and what part is played by other activated cells that have the capacity to secrete chemokines such as monocytes, macrophages, and astrocytes (9–11) and the capacity to present CNS antigens.

We have recently constructed an immunotoxin (IT) composed of an anti-rhesus monkey CD3 monoclonal antibody (mAb), FN18, and a binding site mutant of diphtheria toxin (DT), CRM9 (12). This immunotoxin, FN18–CRM9, is an analogue of UCHT1–CRM9, an anti-human CD3 immunotoxin which is capable of regressing established xenografted human T cell (Jurkat) tumors in nude mice via protein synthesis inhibition (13). A truncated toxin engineered fusion protein version of UCHT1–CRM9 has also been constructed that displays resistance to blockade by human anti-DT antibodies arising from immunizations with DT toxoid (14). When FN18–CRM9 was given iv to a group of rhesus

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monkeys, blood and lymph node T cells were depleted within 48 h in a dose-dependent manner to levels as low as 1% of initial values. Other blood cells were not affected. This resulted in a marked population inversion of T and B cells in the blood and lymph node compartments. T cell repopulation then occurred and was rapid in juvenile animals (2–4 weeks) and slower in older animals (12). Monkeys treated in this manner become tolerized to mismatched renal allografts (15). This novel immunotoxin enabled us to investigate EAE progression in the absence of the peripheral T cell population that experienced immunization with the encephalitogenic MBP. In some monkeys in this study we combined IT with cranial irradiation for the purpose of inducing CNS T cell cytoreduction. This was done because of concern that T cells within the CNS blood–brain barrier sanctuary might not be accessible to blood compartment IT and might not be depleted. We report in this communication that IT-induced transient T cell depletion with or without cranial irradiation markedly attenuates EAE progression and greatly reduces CNS cellular infiltrates, eliminating detectable perivascular infiltrates in some monkeys.

MATERIALS AND METHODS

Animals. Thirteen rhesus monkeys (*Macca mullata*) 4–6 years of age obtained from the Texas Primate Center (Alice, Texas) comprised this study. All were negative for antibodies directed at diphtheria toxin.

Immunotoxin construction. FN18 is a murine IgG1 monoclonal antibody directed at rhesus CD3 and activates T cells in the presence of mixed mononuclear cells (16). CRM9 is a binding site mutant of DT, and has only 1/300 the systemic toxicity of wild-type DT (17). The immunotoxin, FN18–CRM9, was synthesized as previously described by thiolating both FN18 and CRM9 moieties and then crosslinking with bismaleimidohexane. The 1:1 complex used in this study was purified by size exclusion (12).

EAE induction and treatment. Rhesus monkeys were used in accordance with NIH guidelines in a protocol approved by the NIMH ACUC. Monkey brain MBP (lot 95 from African green monkeys) purified by the batch method was kindly provided by G. E. Deibler (18). We modified a protocol kindly supplied by L. M. Rose and E. C. Alvord Jr. in which the ratio of monkey MBP to heat-killed mycobacteria is set at 2:1. Aqueous MBP at 20 mg/ml was emulsified with an equal volume of complete Freund's adjuvant (DIFCO) containing 10 mg/ml heat-killed *Mycobacterium tuberculosis* (H37 RA, DIFCO Laboratories, Detroit, MI). Each rhesus monkey was immunized with intradermal injection of 0.2 ml emulsion distributed between two sites above the ankle on both hind legs. In 12 monkeys cerebrospinal fluid (CSF), 1.5-ml samples, was collected by puncture into the subarachnoid space at the cisternal magna. This was done once a day for 5 consecutive days starting from 11 days after the immunization. The 13th monkey was used to provide lymph node T and B cell data pre- and post-IT.

Four monkeys were used as nontreated controls, 4 were treated with FN18–CRM9 alone, and 4 were treated with FN18–CRM9 in conjunction with cranial irradiation. The first 8 monkeys were immunized as pairs and were randomly assigned prior to immunization to either the nontreatment group or the FN18–CRM9 treatment group. FN18–CRM9 was administered to monkeys in 2 split doses by intravenous bolus infusion at a total amount of 0.2 mg/kg. The first dose of the immunotoxin was given to monkeys on the same day when an increase in CSF WBC > 30 cells/μl was detected. This day was considered as Day 0. The second dose of FN18–CRM9 was infused on Day 2. Radiation was given in one fraction of 650 cGy (monkeys 1144, 1109), one fraction of 325 cGy (1181), or two daily fractions of 325 cGy (1106) using opposed lateral shaped X-ray fields generated by a linear accelerator. The radiation was delivered to anesthetized air-breathing animals on Day 1 following CSF pleocytosis (1109, 1181, 1144) or Day 3 (1106) and covered the entire cranial vault down to C2 with shielding of the orbit, oral cavity, pharynx, and most of the parotid. The dose rate was ≈100 cGy/min. Monkeys with EAE were monitored at least twice each day. The disease activity was scored as 0–5 using an EAE scoring system described previously by Massacesi and co-workers (3) and detailed in the legend to Fig. 1. EAE monkeys were euthanized under the following conditions: 1, obvious neurologic signs such as ataxia and tremors lasting 2 days or an inability of self feeding; 2, one or more limbs paralyzed; 3, 60 days after the immunization. Monkeys were euthanized by intravenous injection of 3 ml euthanasia-5 solution (Veterinary Laboratories Inc., Lenexa, KS). Brains and brain stems were surgically removed immediately following euthanasia and saline perfusion through the left ventricle and stored at −80°C for histologic examination.

Flow cytometry. Both blood and CSF samples were analyzed with flow cytometry utilizing a lysis method (12). The following mAb reagents that have been demonstrated to be reactive to rhesus monkey lymphocytes were used (19): fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated mAbs reactive with the lymphocyte surface antigens CD3 (FN18–FITC, this lab), CD20 (B1-PE, Coulter Corp., Hialeah, FL), CD4 (OKT4–FITC, Ortho Diagnostic Systems Inc., Raritan, NJ), CD8 (DK25–PE, DAKO Corporation, Carpinteria, CA), CD2 (T11–PE, Coulter), CD16 (3G8–FITC, AMAC, Inc., Westbrook, ME), CD25 (IL-2R1–PE, Coulter), CD45RA (2H4–PE, Coulter).

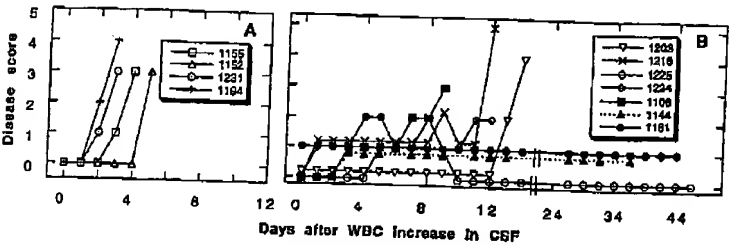

FIG. 1. EAE disease process in nontreated (A) and treated monkeys (B). EAE was scored following a scoring system (3): 0, normal neurologic exam; 1, lethargy, anorexia, weight loss; 2, ataxia, tremor; 3, blindness, paraplegia, hemiplegia; 4, quadriplegia, quadriparesis; and 5, moribund. Day 0 means the time when WBC increase in CSF was detected for the first time. Monkeys 1203, 1216, 1225, and 1224 were treated with FN18–CRM9, and 1106, 1144, and 1181 (filled symbols) were treated with FN18–CRM9 and cranial irradiation.

Immunohistochemistry. Immunohistochemical staining was carried out on frozen brain sections using a previously described protocol (20) employing a secondary anti-mouse horseradish peroxidase antibody and diaminobenzidine (Sigma) chromagen. Primary mAbs used were all of mouse origin: anti-human vimentin (1:50 dilution, DAKO), anti-human MHC class I (W6/32) hybridoma supernatant, anti-human HLA-DR (1:50 dilution, Becton–Dickinson), anti-human CD11b (1:50 dilution, Serotec), anti-human CD68 (1:100 dilution, DAKO), anti-rhesus CD3 (FN18 3 mg/ml 1:100 dilution) (16), anti-human Thy 1 (1:50 dilution, Chemicon), anti-human CD4 (1:25 dilution, Ortho Diagnostic Systems Inc.), anti-human CD8 (1:50 dilution, DAKO). Both primary and secondary antibodies were applied in rhesus sera diluted 1:5 in bovine serum albumin.

RESULTS

Increased WBC in CSF predict the clinical onset of EAE. All monkeys (with the exception of 964) were monitored by CSF examination via the cisterna magna for 5 days starting 11 days postimmunization. This procedure was well tolerated and in itself did not induce pleocytosis (>30 cells/μl). Four of these monkeys were used as nontreatment controls, and the rest were treated at the time of the first detectable WBC increase in CSF. Among the 4 control monkeys clinical EAE occurred 2–6 days after the observed WBC increase in CSF, approximately 15–20 days after the immunization (Fig. 1A). The early symptoms of EAE included apathy, loss of appetite, and weak hind limbs, with rapid deterioration to paralysis.

The first CSF samples displaying increased WBC in all 12 monkeys contained 31–700 cells/μl (normal range 0–7 cells/μl), including monocytes, eosinophils, lymphocytes, and neutrophils. In half of the samples eosinophils occupied a high percentage, 22–78%. In contrast, this high percentage of eosinophils never occurred in the blood (<6%). In samples without such a high percentage of eosinophils, monocytes predominated proportionally, ranging from 42 to 81%. Lymphocytes were about 10–25%, and neutrophils usually below 10%. The lymphocyte population was mainly composed of T cells (60–90%) in the FACS analysis. A small proportion of B cells (<5%) was also observed. Both $CD4^+$ and $CD8^+$ cells were present, with the former having a slightly higher percentage. $CD3^+CD25^+$ cells ranged from 5 to 24% in these CSF samples, which was 4 times higher than their counterparts in the blood (data not shown).

T cell depletion slows down the disease progress. FN18–CRM9 transiently depletes T cells in rhesus monkeys (12). This also occurs in MBP immunized monkeys as shown in Figs. 2 and 3 by FACS analysis on blood and macerated lymph node taken before and after IT treatment. $CD3^+$ cells in the blood decreased

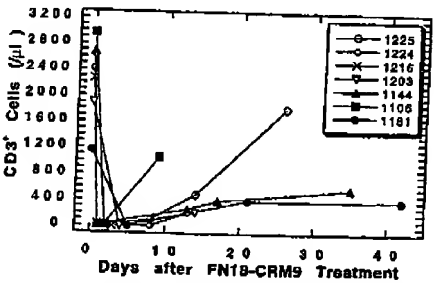

FIG. 2. T cell depletion and repopulation in blood of monkeys treated with FN18–CRM9 by FACS. The first day of FN18–CRM9 treatment is recorded as Day 0.

to very low levels compared with that before IT treatment ($P < 0.01$). These low levels of T cells remained for about 10–14 days. Note also the marked inversion of the lymph node T/B cell ratio.

Previous studies showed that EAE in rhesus monkeys had an acute onset and caused death within days (5, 6). This is confirmed by our data. As shown in Fig. 1A, EAE in all 4 nontreated control monkeys reached grade 3 disease within 3 days after the onset and required euthanasia. Of the 8 experimental monkeys, 4 were treated with intravenous infusion of FN18–CRM9, and the remaining 4 had cranial irradiation in addition to FN18–CRM9. Survival status of these monkeys is depicted in Fig. 1B. The 4 monkeys treated with FN18–CRM9 survived from 11 to 45 days before euthanasia, significantly longer than the control monkeys ($P < 0.01$). One of them, 1225, survived disease free, and was euthanized subject to the 2 months time schedule in the protocol. In the 4 monkeys treated with FN18–CRM9 plus cranial irradiation, one died several hours after the irradiation, possibly due to irradiation-induced acute CNS syndrome. Therefore, this monkey is not included in the analysis. The other 3 monkeys experienced vomiting, dyspnea, and tremors within 30 min postirradiation. Two of them also developed a facial cellulitis that lasted for 7–20 days. However, these 3 monkeys survived the early side effects of cranial irradiation and the cellulitis, and lived on for 10 to 44 days before euthanasia. Among over 50 rhesus monkeys treated to their whole body with a total dose of 1300 cGy in two daily fractions for bone marrow transplantation studies at this center, there have been no acute deaths, no evidence of seizure activity, no parotidis, or evidence of facial cellulitis.

EAE progress to stage 2 or 3 disease in the treated monkeys was either retarded (1224, 1203, 1216, and 1106), or halted to a condition showing only minor symptoms such as loss of appetite, decreased activity, and weight loss (1144 and 1181) or no symptoms (1225).

T cell repopulation. Blood and lymph node T cells remained at a very low levels for about 7 days after

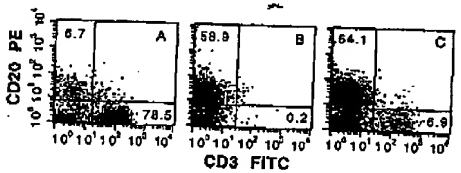

FIG. 3. Lymph node lymphocyte population phenotypes before (A) and 4 and 11 days after FN18–CRM9 (B and C, respectively) by FACS in a single EAE monkey (964). The immunotoxin induces a marked inversion of the normal T/B cell ratio (lower right/upper left quadrants).

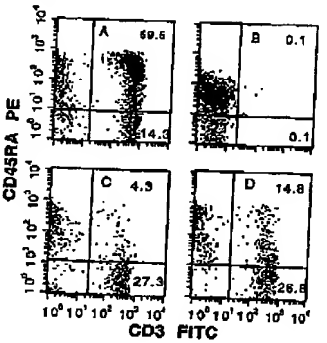

FIG. 4. $CD3^+CD45RA^-$ cells repopulate earlier than $CD3^+CD45RA^+$ cells in blood of FN18–CRM9-treated monkeys. Blood samples were taken 1 day before immunotoxin treatment (A) and after treatment on Days 1 (B), 17 (C), and 41 (D) and analyzed with flow cytometry (data from monkey 1181).

treatment, and then started to repopulate (Figs. 2 and 3). In the 3 long-term survivors (Fig. 1B), only one (1225) repopulated blood T cells to a level close to that before treatment, and the other 2 failed to achieve a complete repopulation. $CD4^+$ and $CD8^+$ T cells repopulated simultaneously in most of the monkeys (data not shown). Comparing the T cell data in the blood between the monkeys treated with FN18–CRM9 only and those treated with FN18–CRM9 plus cranial irradiation, no obvious difference was observed (Fig. 2). T cell repopulation in the blood was consistently initiated by $CD3^+CD45RA^-$ cells, and only until about 25 days after the treatment did the $CD3^+CD45RA^+$ cells appear in a significant proportion (Fig. 4).

CSF T cell numbers also dropped quickly after IT treatment as did granulocytes. In 4 monkeys where data are available CSF T cells decreased to 1–3% of initial values after immunotoxin treatment. Most of this change occurred in the first 48 hr. Unlike T cells in the blood, CSF T cells remained at low levels in the follow-up period (Fig. 5B). In contrast, T cells in nontreated monkeys increased severalfold within days (Fig. 5A).

The inflammatory response at the immunization sites of the treated monkeys fluctuated in accordance with the blood T cell status. The injection sites became red and swollen 1 day after the immunization, and the involved area was about 0.8 × 0.8 cm. This remained until Day 9 when the inflammatory area enlarged to approximately 2.0 × 1.5 cm, and became ulcerated in the middle. This severe local reaction started to resolve 1 day after the first dose of FN18–CRM9 and ulcers sealed within 3 days. However, the inflammation re-

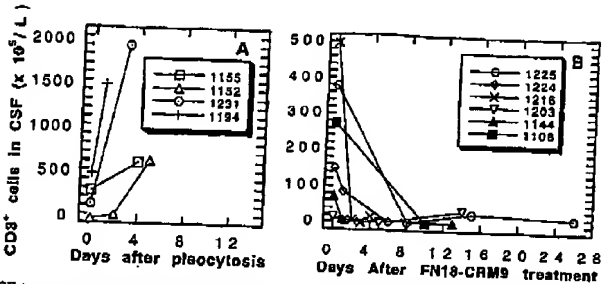

FIG. 6. T cells in CSF increased in nontreated EAE animals (A) and were depleted in EAE monkeys treated with FN18–CRM9 (B). Day 0 is either the date of detecting the first CSF sample with increased WBC (A) or the first FN18–CRM9 treatment (B).

turned at the same area, and ulceration reoccurred in monkeys with repopulated T cells (1225, 1144, 1181).

Immunohistochemistry. As seen in Table 1 and in Figs. 6A–6E, the nontreatment EAE cohort had numerous large perivascular inflammatory plaques throughout the white matter of the hemispheres easily identified in sections stained with specific markers or with hematoxylin alone. These plaques were remarkable for marked up-regulation of vimentin in the parenchyma adjoining the plaques, consistent with acutely reactive astrocytes. The parenchyma was also notable for up-regulation of both MHC class I and II (MHC class I > MHC class II). Although double staining was not done, the MHC class I expression is probably on microglial cells and astrocytes given the diffuse nature of the staining. The MHC class II expression is more restricted and probably represents expression by microglial cells. The inflammatory infiltrate was notable for marked macrophage infiltration as seen by the extraordinary CD68 (Fig. 6A) and CD11b expression. $CD4^+$ and $CD8^+$ cells also constituted a significant population of cells in the infiltrates, further confirmed by the staining for CD3 (Fig. 6B). MHC class I and class II expression in these inflammatory plaques was also quite remarkable (Figs. 6C and 6D), again class I expression being greater than class II expression.

In contrast to the untreated EAE cohort, the cohort treated with FN18–CRM9 or FN18–CRM9 plus irradiation had no identifiable inflammatory plaques in the hemispheres in 4/5 treated monkeys subjected to histochemistry (Figs. 6F–6J and 6K–6O). However, small and large vessels often exhibited enhanced CD68 staining (Fig. 6K), and occasional plaques were seen in the brain stem (Figs. 6P and 6Q). Rare to no $CD3^+$, $CD4^+$, or $CD8^+$ cells could be identified in these infiltrates (Fig. 6Q). The plaques in the fifth monkey consisted predominately of CD11b- and CD68-expressing macrophages (Figs. 6R and S). Again, few $CD3^+$, $CD4^+$, or $CD8^+$ cells could be identified in these infiltrates (Fig. 6S). The brain parenchyma of immunotoxin-treated animals euthanized before 15 days of active disease dem-

TABLE 1
Summary of Histopathology

Monkey	Therapy	Disease duration days	Terminal disease score	Plaques brain	Plaques brain stem	CD68	CD3	CD4	CD8	MHC I	MHC II	Vimentin
1231	—	4	3	62 ± 11	nd	4+	3+	3+	3+	4+	4+	4+
1134	—	6	4	42 ± 6	nd	4+	3+	2+	3+	4+	4+	4+
1208	IT	14	4	<1	nd	r	—	—	—	bv	—	—
1106	IT + R	9	3	<1	3 ± 0	3+	—	—	—	4+	2+	2+
1225	IT	45	0	<1	nd	r	—	—	—	bv	—	4+
1181	IT + R	44	1	<1	<1	r	r	—	—	bv	—	4+
1144	IT + R	35	1	68 ± 10	16 ± 5	4+	1+	—	—	4+	3+	4+

Note. IT, immunotoxin; IT + R, immunotoxin + cranial irradiation; bv, blood vessels only; nd, not done. Percentage of cells in plaque staining for antigen: —, 0%; r, rare isolated cell; 1+, 1–10%; 2+, 10–33%; 3+, 34–65%; 4+, 67–100%. Plaque quantitation: 3 noncontiguous sections covering at least 10 cm^2 were scanned under low power, plaques were counted, averaged and are reported per 10 cm^2 of tissue.

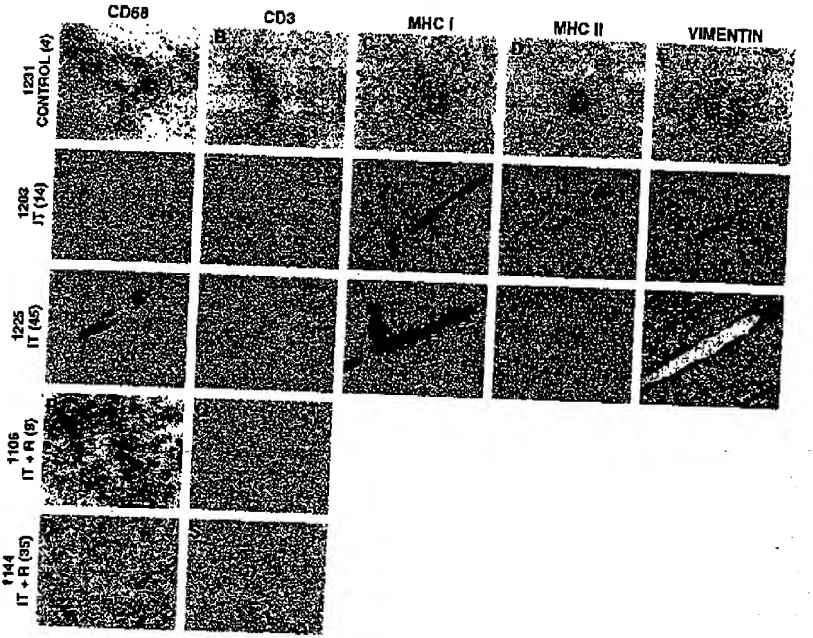

FIG. 6. Immunohistochemistry. Rows display contiguous sections of brain or brain stem that were stained for cell surface epitopes by secondary immunoperoxidase methodology and counterstained with hemotoxylin. Positive epitope staining is brown. Epitope staining is delineated by the column heading and is constant by column. Rows are signed by the monkey number, the treatment (as in Table 1) and the disease duration in parentheses. When identifiable the field covers the same histological structure, otherwise the field is from the same general area of the section. A–E shows a typical perivascular plaque in a nontreated EAE monkey brain. Perivascular plaques were not observed in most treated monkey brains (F–J and K–O) but were seen in the brain stem (P–Q and R–S). However, these plaques while containing macrophages (P and R) were largely devoid of CD3$^+$ T cells (Q and S) in contrast to untreated EAE monkeys (B). Printed magnification is 100×. See Results for detailed comments.

onstrated little gliosis as seen by vimentin staining (Fig. 6J) in contrast to animals surviving 35–45 days where gliosis was marked (Fig. 6O).

DISCUSSION

Nonhuman primate models of EAE have the advantage of sharing considerable similarity with the human immune system. We chose to evaluate anti-CD3 immunotoxin as a treatment for a T-cell-driven autoimmune diseases in rhesus monkeys because of the availability of the anti-rhesus anti-CD3 antibody FN18. We induced a highly consistent monophasic form of the disease having a narrow window between immunization and disease onset by using a 2:1 ratio of monkey MBP to heat-killed mycobacteria. This relatively small window allowed us to predict EAE onset by performing serial CSF WBC and FACS analyses. CSF pleocytosis occurred 1–5 days before the appearance of clinical signs in 10 of 11 monkeys, and allowed us to initiate early treatment. This method of early prediction of subsequent EAE is much simpler than MRI. It is based on the observation that in rodent EAE the meninges and subarachnoid space are infiltrated with inflammatory cells before brain and spinal tissue (21) and in some models before the onset of neurological signs (10). We reasoned that if this were the case in rhesus EAE, CSF pleocytosis should likely precede clinical EAE. The consistency of this model permits therapeutic evaluations with a relatively small group of monkeys and is in our

opinion preferable to EAE treatment evaluations where treatment is initiated prior to any signs of disease onset (9, 11).

Treatment with immunotoxin lowered CSF pleocytosis to near normal levels within 3 days. Treatment also dramatically prolonged the survival time of EAE monkeys. In nontreated monkeys EAE progressed rapidly, and these monkeys required euthanasia within 3 days after EAE onset having reached a disease score of 3 or greater. Thus there was a striking negative correlation between the average CSF T cell count and the survival time ($R = 0.93$) in nontreated monkeys but not in treated monkeys ($R = 0.24$), indicating that treatment attenuated a major correlate of the disease process. Histopathology on these nontreated monkeys showed numerous brain perivascular plaques containing T cells and macrophages. In the treated monkeys EAE manifested either as a delayed acute process (survival time was prolonged to 2 weeks), or as a mild form, reflected by loss of appetite, weakness, and weight loss (survival time was 35 days or the last day of the protocol, Days 44–45). Histopathology was performed on two monkeys euthanized during a delayed acute process. The hemispheres of these brains were nearly normal. However, the brain stem of the animal treated with immunotoxin plus radiation displayed a single plaque devoid of identifiable T cells and mild gliosis. The occasional brain stem plaque could have been responsible for the clinical deterioration seen in the delayed acute-phase animals. However, the observed plaque was not centered on an intact vessel and may represent an old, rather than a new lesion. Two of the three animals euthanized between Days 35 and 45 during the mild form of the disease and subjected to histopathology failed to show plaques. However, all of these animals exhibited marked gliosis. The third treated monkey exhibiting a clinical mild form of the disease showed about the same number of perivascular plaques seen in nontreated animals but T cells were infrequent. Again, marked gliosis was present. It is not clear whether this gliosis represents a late response to the early acute disease or whether it represents an ongoing chronic process. No differences were apparent between the radiation and nonradiation treatment groups.

Therapies previously shown to prolong survival time in nonhuman primate EAE include treatment with corticosteroids or antibiotics plus high dose MBP (22), monoclonal anti-CD4 (5, 23), and a monoclonal antibody cross-reactive with RhLA II (6). Although there was some reduction noted in the histopathology in these treated animals, major changes compared to nontreatment groups were not noted (5, 6, 23, 24). In particular, perivascular cellular infiltrates, the hallmark of new lesions, contained T cells after treatment. In contrast, immunotoxin treatment produced a reduction in the number and size of new perivascular brain lesions. The striking feature was that these lesions when present were largely devoid of T cells.

In 4 monkeys where data are available CSF T cells decreased to 1–3% of initial values after immunotoxin treatment. Most of this change occurred in the first 48 hr. The decline of T cells might be due to a leak of IT into the CSF through partial disruption of the blood–brain barrier, but this is unlikely since we could not detect therapeutic levels of IT in the CSF as we could in blood by ELISA (data not shown). More likely these T cells have a short CNS half-life either due to cell death or recirculation to the venous system along with the bulk CSF. Their decline indicates that they are not being replaced. The reduction of brain T cell and macrophage infiltrates seen in treated animals compared to untreated animals also probably occurs through lack of replacement since in self-limiting rodent EAE these infiltrates regress within 10 days (25). The residual macrophage infiltrates that are seen in the treatment groups may represent continued macrophage infiltration driven by residual vascular damage or a very small number of residual T cells. These could have survived immunotoxin therapy due to the sanctuary provided by the blood–brain barrier. This was the reason for providing an IT plus cranial irradiation group in this study. Since activated T cells are radiation sensitive with a D_0 estimated to be 52 cGy (13), 325 cGy could achieve a 1.8 log reduction of this cell type. However, in this limited study we could not detect a significant difference between IT alone and IT plus radiation. A trend toward longer survival in the group receiving radiation may or may not be real. It is possible that early radiation may inactivate CNS T cells more quickly than IT which probably works by preventing reseeding. Early radiation-induced inactivation of CNS T cells might limit CNS damage in this acute disease process.

Animals in this study were hypersensitive to acute radiation-induced phenomenon. Whole brain radiation at the doses delivered is associated with only rare nausea or transient asymptomatic parotid swelling in human subjects, a negligible rate of seizure, and no deaths (26). Likewise, whole body radiation to other rhesus monkeys in our laboratory, using a dose two to four times the dose used in this study has resulted in no acute deaths, seizures, or parotidis. The frequency of facial swelling, seizure activity, and probably CNS syndrome seen in the current study suggests that EAE (or immunotoxin) predisposed irradiated cells in the brain and probably to the salivary gland to depolarize (27), apoptose (28), or release cytotoxic factors (29) that caused edema and/or necrosis.

Most of the treated monkeys surviving at 12–21 days had begun to repopulate their blood T cells by this time to a range of 250–500 cells/μl. By 3 weeks the inflammatory lesions at the immunization sites which had regressed 48 hr after IT treatment showed a recrudes-

cence of inflammation and began to ulcerate again. One monkey, 1225, was tested for PPD sensitivity at Day 41 and was strongly positive. These findings indicate that the repopulated T cells could exhibit delayed-type hypersensitivity reactions. Mycobacterial antigens were continually present since the draining immunization sites continued to shed heat-killed acid fast bacteria from the adjuvant. However, T cells did not apparently repenetrate the blood–brain barrier in large numbers as evidenced by repeated CSF FACS analysis and histopathology at the time of euthanasia.

The difference in behavior of repopulated T cells toward tissue depots containing mycobacterial antigens versus CNS MBP may reflect the very low level or absence of peripheral MBP at the time of T cell repopulation. In rat EAE bromodeoxyuridine labeling has shown that most of the MBP-specific T cell proliferation occurs in the periphery as opposed to the CNS (30). The severity of the initial disease is also very sensitive to the MBP dose (5). This suggests that in active EAE the number of peripheral MBP-reactive T cells is a major variable that determines the magnitude of the CNS cellular infiltrates and the severity of the disease, a fact demonstrated in passive EAE (31). It seems reasonable to speculate that the postimmunotoxin-repopulated T cell population contains a greatly reduced number of MBP-reactive effector cells compared to the population at the time of treatment. We think that this is the most likely mechanism to explain the beneficial effect of immunotoxin at late times following T cell repopulation. This could reflect a decrease in the number of T cells carrying TCR specific for MBP. However, there are other possible mechanisms that might be operative during the T cell depletion and subsequent repopulation phases. Part of the effect could be due to alterations in the functions of the repopulated T cells capable of binding MBP. For example, the naive phenotype $CD3^+$ $CD45RA^+$ is very underrepresented in the early repopulation period. Alternatively, repopulation during the disease process might induce a switch (immune deviation) in phenotype from T_H1 to T_H2 that would give the appearance of tolerance to MBP (32). Anti-CD3 immunotoxin has been shown to induce tolerance to rhesus renal allografts mismatched at both MHC class I and class II loci. Tolerance was shown by the acceptance of donor skin grafts and the rejection of third party grafts. This appears to take several months to develop indicating an active immune process (15). However, the mechanism of this process has not yet been delineated. One possible mechanism is that during the depletion phase the ratio of non-professional antigen-presenting cells (B cells) greatly exceeds the number of T cells. It has been suggested that this population inversion which is akin to the neonatal state favors tolerance over immunization (33). Whether a similar tolerizing processes to autoimmune-inducing antigens could be operative in the immunotoxin toxin treatment of autoimmune diseases remains to be elucidated.

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